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Short communication

Comparison of chloroform fumigation-extraction, phospholipid fatty acid, and DNA methods to determine microbial biomass in forest humus

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Abstract

Techniques developed to measure microbial biomass in mineral soils may not give reliable results in humus. We evaluated the relationships between three techniques to estimate microbial biomass in forest humus: chloroform fumigation-extraction (CFE), total extractable phospholipid fatty acids (PLFA), and extractable DNA. There was a good relationship between PLFA and CFE ($R^2 = 0.96$), with a slope slightly different from that previously reported for mineral soils (1 nmol PLFA corresponded to a flush of 3.2 μg C released by fumigation in humus cf. 2.4 μg C in mineral soil). There was no relationship between DNA concentration and the other two measurements of microbial biomass. This may be due, in part, to the high fungal biomass in forest humus, as DNA concentration per unit biomass is much more variable for fungi than bacteria.
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Soil microbial biomass is difficult to measure and it is largely through agreements among different methods that we gain confidence in new measurements (Martens, 1995). Modifications of some methods are needed to achieve reliable estimates in different soil types due to chemical characteristics of the soil or the composition of the soil community (Sparling et al., 1990; Alphei et al., 1995; Anderson and Joergensen, 1997). Forest humus, in particular, with its low pH, high organic matter, and variable ratios of fungal-to-bacterial biomass, has proven to be problematic for many techniques. Total phospholipid fatty acids (PLFA) are now routinely used to estimate soil microbial biomass, and DNA extracted for molecular analyses has been proposed as another measure (Marstorp et al., 2000). PLFA can also be used to estimate the ratio of fungal-to-bacterial biomass (Frostegård and Bååth, 1996). These biomass measures have been compared to the more standard technique of chloroform fumigation-extraction (CFE) in mineral soils (Marstorp et al., 2000; Bailey et al., 2002), but not in forest humus. Here, we address whether relationships among chloroform-labile carbon, PLFAs, and DNA established for mineral soil apply to forest humus.

These data were collected as part of an investigation of humus microbial communities in two forest types on northern Vancouver Island, British Columbia that have substantially different rates of nitrogen mineralisation and nitrogen availability (Leckie et al., in press). Western redcedar (*Thuja plicata* Donn.)–western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) (CH type) and western hemlock–amabilis fir (*Abies amabilis* (Dougl.) Forbes) (HA type) forests have large forest floor accumulations (up to 1 m deep) and are described by Prescott and Weetman (1994). In October 2001, adjacent CH and HA stands were found at four locations. Ten samples were composited from each of the F, upper humus (H_U), and lower humus (H_L) forest floor layers from each of the eight stands. Samples were sieved to <2 mm and duplicate subsamples were dried at 70 °C for 24 h to determine moisture content. All biomass measures are reported on a dry matter basis.

The chloroform-fumigation extraction method was conducted using 30 g (fresh weight) of each sample according

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Table 1
Moisture, pH, CFE-flush, PLFA, and DNA concentrations in F, upper humus (H_U), and lower humus (H_L) layers of cedar-hemlock (CH) and hemlock-amabilis fir (HA) forest floors. Values are the mean and standard error of the mean of four sites

	Moisture (% of wet weight)	pH	CFE (mg C g^{-1} dry humus)	Total PLFA (nmol g^{-1} dry humus)	DNA (ug g^{-1} dry humus)	Fungal PLFA (nmol g^{-1} dry humus)	Fungal-to-bacterial PLFA
CH forest							
F	76.1 (1.3)	4.37 (0.09)	4.27 (0.88)	1735 (117)	382 (74)	170 (5)	0.29 (0.01)
H_U	78.4 (1.3)	3.92 (0.08)	2.88 (0.19)	1355 (71)	413 (53)	96 (5)	0.21 (0.03)
H_L	80.3 (1.1)	3.79 (0.09)	1.84 (0.07)	886 (53)	455 (60)	44 (6)	0.14 (0.02)
HA forest							
F	76.9 (1.0)	3.92 (0.03)	3.75 (0.40)	1541 (48)	461 (68)	152 (15)	0.31 (0.03)
H_U	77.8 (1.0)	3.64 (0.04)	2.77 (0.25)	1241 (35)	490 (33)	70 (7)	0.16 (0.02)
H_L	77.4 (1.0)	3.71 (0.09)	1.49 (0.17)	968 (73)	490 (49)	28 (4)	0.07 (0.02)

to the standard technique (Vance et al., 1987; Tate et al., 1988). Fumigated and unfumigated samples were extracted with 0.5 M K_2SO_4, gravity-filtered with presoaked Whatman 42 filter paper, and then vacuum-filtered with 0.45 μm Millipore filters. Organic carbon was analyzed using the high-temperature combustion method, with the Shimadzu TOC-500 Carbon Analyzer. Microbial biomass carbon was estimated as the difference between fumigated and unfumigated samples and no correction factor was used.

DNA was extracted and purified from equivalent dry weights of each sample (400–500 mg fresh weight), using the Bio 101 Fast DNA Kit for Soil (La Jolla, California), with some modifications from the manufacturer's directions based on empirical determination as described below. This method involves direct extraction of DNA from the humus after mechanically lysing cells with quartz beads in a buffer solution. Samples were vigorously shaken using a Mini Bead-Beater (BioSpec Products, Bartlesville, Oklahoma) for 2.5 min at 5000 beats per minute and then centrifuged for 10 min (14,000 rpm, Eppendorf Centrifuge 5415C). 100 μl of the supernatant was drawn off and DNA was purified with a protein precipitation and washed twice with ethanol–salt solution in spin filter columns. DNA was eluted in 100 μl of ultra-pure water. Purified DNA was quantified on a 1% agarose gel, using ethidium bromide as a nucleic acid-binding stain and imaged using an AlphaImager 1200 (Alpha Innotech, CA). A range of known amounts of standard 1 kb DNA ladder were used to construct a calibration curve using AlphaEase (version 3.3, Alpha Innotech, 1996). DNA concentrations were then scaled up to calculate the total amount of (purified) DNA that was present per gram of dry humus.

Lipids were extracted from approximately 650 mg (fresh weight) humus using a Bligh and Dyer (1959) extraction, as modified by White et al. (1979) and Frostegård et al. (1991). The polar lipid fraction was subject to a mild alkaline methanolysis to yield fatty acid methyl esters (FAMEs). FAMEs were separated and quantified by gas chromatography (Hewlett Packard E5895 Series II) using splitless injection, helium as a carrier gas, and a polar column, and were identified compared to standards. Fatty acid nomenclature follows Frostegård et al. (1993). Fatty acids with less than 20 carbons were included in the calculation of total PLFAs. Fatty acids 16:1ω7c, 16:1ω7t, cy17:0, and 18:1ω7, i15:0, a15:0, i16:0, a17:0, br17:0, 15:0, and cy19:0 were used to represent bacterial biomass (Federle, 1986; Lechevalier and Lechevalier, 1988; Frostegård et al., 1993). The fatty acid 18:2ω6,9 was used to represent fungal biomass (Federle, 1986; Frostegård and Bååth, 1996).

Microbial biomass carbon estimates and total PLFA decreased in deeper layers of the forest floor (Table 1). A significant positive relationship was found between microbial biomass carbon, as estimated by CFE, and total PLFA ($R^2 = 0.96$, $p = 0.0007$) (Fig. 1). Bailey et al. (2002) found a strong relationship between these two measures for mineral soils and proposed the following general equation to convert PLFAs to the more common measure of microbial carbon

$$CFE_{flush} = 2.4(totPLFA) + 46.2$$

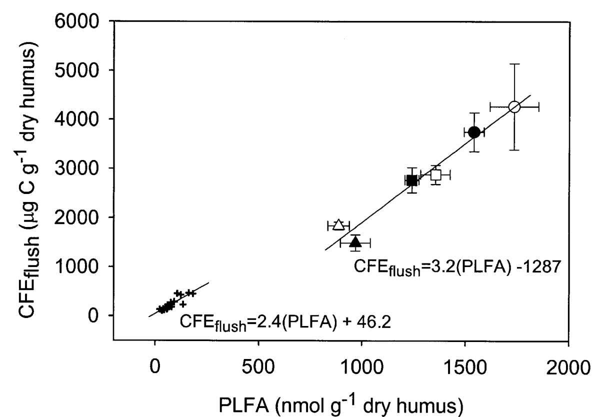

Fig. 1. Relationship between the chloroform fumigation-extraction (CFE) estimate of microbial biomass carbon and total microbial PLFA. Circles are F-layer samples, squares are upper humus layer samples, and triangles are lower humus layer samples. Symbols are mean and standard error of the mean of four sites of cedar-hemlock (unfilled) and hemlock-amabilis fir (filled) forests. Small crosshairs are mineral soil data from Bailey et al. (2002).

Their measure of total PLFAs differs slightly from ours; for monounsaturated PLFAs, only those with less than 18 carbons were included by them. Although that study included a range of mineral soils, values of biomass were all an order of magnitude smaller than the values reported here for humus (Table 1; Fig. 1). Humus is much lighter than mineral soil and it is difficult to interpret differences expressed on a dry mass basis. The linear relationship found in this study

$$CFE_{flush} = 3.2(totPLFA) - 1287$$

is significantly different ($p < 0.0001$) from that found by Bailey et al. (2002). This equation may be more appropriate for forest humus, complementing the relationship demonstrated by Bailey et al. (2002) for mineral soils.

DNA yield, however, did not correlate with microbial biomass carbon ($p = 0.16$) or total PLFAs ($p = 0.15$). Marstorp et al. (2000) found a strong correlation between microbial biomass carbon, estimated by CFE, and extracted DNA and suggested that DNA could be used as a measure of microbial biomass. However, their study was on agricultural soils with less than 3% organic matter and low total microbial and fungal biomass compared to forest humus. Griffiths et al. (1997) found no relationship between DNA and microbial biomass C in mineral soils incubated with heavy metals. They found increases in extracted DNA per unit carbon in treatments that had lower fungal biomass (based on PLFA). The DNA content of fungi per unit biomass is both lower and much more variable than that of bacteria (Harris, 1994), and thus DNA content seems to be a poor measure of biomass for fungi. In this study, total PLFA, fungal PLFA, and the ratio of fungal-to-bacterial PLFA, decreased in deeper forest floor layers (Table 1). Coniferous forest humus typically has high fungal biomass and the ratios of fungal-to-bacterial PLFA for the F and H_U layers are similar to values reported by Frostegård and Bååth (1996). Therefore, it is possible that the similar DNA concentrations among the three forest floor layers result from the combined effect of differences in total biomass and community composition. Additionally, DNA extraction efficiency may have differed among the samples, due to differences in community composition and the physical and chemical nature of the humus types. In particular, recovery of DNA from gram-positive bacteria and fungi may be lower than that from gram-negative bacteria. DNA extraction methods have been compared for a range of mineral soils (Zhou et al., 1996; Frostegård et al., 1999; Miller et al., 1999; Martin-Laurent et al., 2001), but not for forest humus. We conclude, therefore, that purified, extracted DNA may not be an appropriate measure of total microbial biomass in forest humus.

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